Function of Chikungunya Virus Structural Proteins

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Introduction

Chikungunya virus (CHIKV) belongs to the genus *Alphavirus* (within the family *Togaviridae*), which includes over 29 species that may cause encephalitis, febrile illness, and arthralgia in humans. CHIKV has a single-stranded, positive sense RNA genome of approximately 12 kb in length, although some size variation exists between different lineages. The icosahedral virions have a diameter of 60–70 nm and consist of a nucleocapsid enveloped by a host-derived phospholipid membrane. The viral structural polyprotein is translated from a ~5 kb subgenomic mRNA and is co- and post-translationally cleaved into capsid protein (C), two major envelope glycoproteins (E1, E2), and three smaller accessory proteins (E3, 6K, and the transframe protein TF). Together, the structural proteins encapsidate the viral genomic RNA to form the viral progeny. The different functions of the individual CHIKV structural proteins in virion assembly, egress, binding, and fusion are reviewed in this chapter.

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Chikungunya Virus Capsid Protein

The CHIKV nucleocapsid has a $T=4$ icosahedral symmetry and consists of the viral RNA encapsidated by 240 capsid protein copies (Fig. 1a, b). The CHIKV capsid protein has a length of 261 amino acids and an apparent molecular weight of ~30 kDa (Khan et al. 2002), which is small enough for passive transport through nuclear pores. The CHIKV capsid protein is organized into 3 regions (I, II, and III) with separate functions (Fig. 1c) (Hong et al. 2006).

The capsid protein is autocatalytically cleaved off *in cis* from the nascent viral structural polyprotein (C-E3-E2-6K-E1) by its C-terminal serine protease which has a chymotrypsin-like fold (Aliperti and Schlesinger 1978; Choi et al. 1991; Melancon and Garoff 1987; Strauss and Strauss 1994). A catalytic serine S213 and several conserved H139, D145, D161 amino acids are predicted to be involved in this autoprotease activity which resides in region III of the capsid coding sequence (Hahn and Strauss 1990; Khan et al. 2002). After the autocatalytic cleavage of the capsid from the structural polyprotein, the signal sequence for ER translocation of PE2 becomes available at the N-terminus.

The capsid has a poorly conserved N-terminal region with an alleged role in viral RNA assembly via positively charged Arg, Lys, and Pro residues in region I. Region II is involved in the encapsidation of newly synthesized viral genomic RNA, which is packaged with high specificity due to defined RNA packaging signals within the nsP2 gene. Consequently, only full-length genomic RNA (and not viral subgenomic

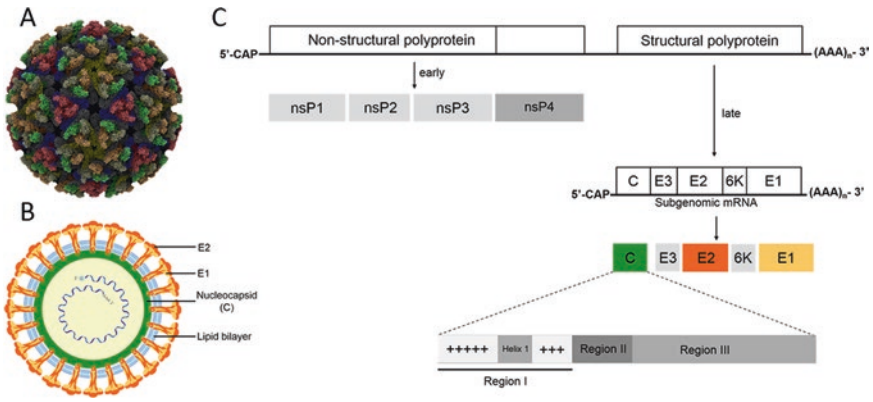

Fig. 1 CHIKV structure and genome. (**a**) CHIKV virion and (**b**) schematic representation of CHIKV particle section (adapted from Metz and Pijlman (2011)). (**c**) Schematic representation of the CHIKV genome, which encodes 2 ORFs. The nonstructural polyprotein encodes nonstructural proteins nsP1, nsP2, nsP3, and nsp4. The structural polyprotein is translated from a subgenomic mRNA and encodes the capsid protein (**c**), envelope glycoproteins E1 and E2 and accessory proteins E3 and **6K**. Capsid is segmented into three separate regions. Region I contains many positively charged residues as well as uncharged amino acids in helix 1. Region II is responsible for RNA binding and region III interacts with the E2 glycoprotein

RNA or host mRNA) is encapsidated in the virions (Owen and Kuhn 1996; Weiss et al. 1989). The capsid protein requires interactions with nucleic acids (i.e., RNA) to initiate assembly.

Alphavirus capsids, including CHIKV, contain an 18 amino acid long coiled-coil α-helix within region I that is important for assembly. Initially, viral RNA-bound capsid dimers are formed, 120 of which further oligomerize to form the nucleocapsid in the cytoplasm of infected cells (Perera et al. 2001). The nucleocapsids are then transported to the plasma membrane for association with the C-terminal cytoplasmic tail of E2 to initiate virion budding. The capsid protein contains a hydrophobic pocket adjacent to the protease substrate binding site within region III for binding to the cytoplasmic endodomain of the E2 glycoprotein, which extends down into the site of the hydrophobic pocket (Kuhn 2007b).

The intracellular localization of the CHIKV capsid is mainly cytoplasmic, although a predicted nuclear localization signal (NLS) is present in the N-terminus (Thomas et al. 2013). Although a putative biological role for a nuclear fraction of CHIKV capsid in viral infection is currently not known, New World alphavirus capsid proteins actively translocate to the nucleus to induce host transcriptional shut-off (Garmashova et al. 2007).

Chikungunya Virus Envelope (Glyco)Proteins

Alphaviruses contain a membraneous envelope that is derived from the infected host cell during budding. Specialized viral glycoproteins embedded in the envelope regulate cell receptor recognition, attachment, and cell entry through fusion of viral with host membranes. CHIKV encodes a single envelope polyprotein comprising four envelope proteins in the order E3-E2-6K-E1. There is also an additional transframe protein (TF) originating from a frameshift event at the 3′ end of 6K (Firth et al. 2008). Not all structural proteins from the envelope cassette are incorporated in progeny virus particles; that is, CHIKV E3 is not generally found associated with virions (Simizu et al. 1984).

The two most conspicuous proteins of the envelope cassette are the glycoproteins E1 (viral fusion protein) and E2 (receptor binding protein). CHIKV-E2 is initially expressed as precursor E2 (E3 E2, or PE2) and matures throughout multiple cellular compartments. Both E1 and E2 are N-linked glycosylated, type I integral membrane proteins that contain transmembrane domains proximate to the C-terminus, followed by a cytoplasmic tail. E1 and E2 heterodimers are exposed at the virion surface as trimeric spikes and consequently are the main targets for a neutralizing antibody response, although E2 is the primary antigen (Strauss and Strauss 1994). In addition to binding cell receptors during the vesicular entry pathway, E2 is essential in intracellular translocation and folding of the other envelope proteins and acts as a stabilizing factor for E1–E2 interactions during transport from the ER to the plasma membrane.

CHIKV E1–E2 Trimer Formation and Virus (-Like Particle) Assembly

The envelope of CHIKV is a multiprotein structure composed of glycoprotein trimeric spikes embedded in a host-derived membrane. The 80 trimeric spikes in the CHIKV virion each consist of three E1–E2 heterodimers and are essential for cell receptor recognition (E2) and entry through pH-dependent endocytosis (E1) (Kielian et al. 2010).

Once capsid is released from its nascent polypeptide chain, the remaining envelope polyprotein or envelope cassette is translocated and inserted into the endoplasmic reticulum (ER) membrane for initial processing (Fig. 2). A series of apolar residues at the N-terminal end of PE2, within the E3 coding region, functions as a signal peptide for the translocation of the envelope cassette into the lumen of the ER (Lobigs et al. 1990). The E3 signal is not processed or removed by host signalases, indicating a distinct role in trimer formation. This was exemplified by the replacement of E3 with an artificial signal peptide that targeted the polyprotein to the ER, but abolished trimeric spike formation and surface expression (Lobigs et al. 1990). The role of E3 in correct translocation and processing of PE2 was also demonstrated by the expression of individual CHIKV-E3E2 protein constructs using both insect and mammalian expression systems (Metz et al. 2011; van den Doel et al. 2014; Voss et al. 2010).

In the ER, proteolytic processing by host signalases cleaves 6K at its N- and C-terminal end, releasing 6K from the envelope polyproteins and yielding PE2, 6K, and E1 (Fig. 2). Immediately after processing, PE2 and E1 form heterodimers, which is followed by glycosylation and the oligomerization of three pE2-E1 dimers to form the immature and nonfusogenic trimeric spike complex (Kuhn 2007a).

Alphavirus E1 and E2 are N-linked glycosylated, but the number of glycans may vary between species (Blom et al. 2004; Burke and Keegstra 1979; Knight et al. 2009; Rice and Strauss 1981; Simizu et al. 1984). CHIKV-E1 is predicted to be glycosylated once at N141 and E2 is glycosylated at N263 and N273 (Blom et al. 2004).

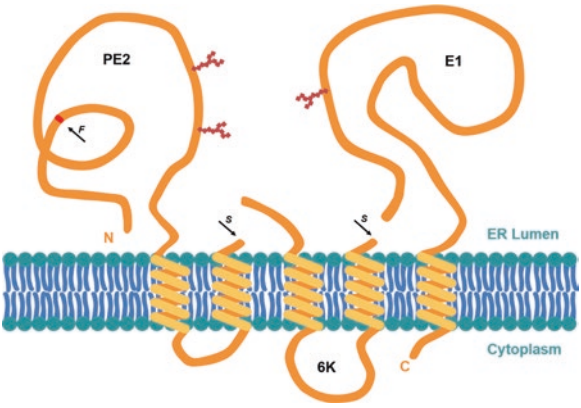

Fig. 2 CHIKV envelope glycoprotein organization. After capsid is released, the envelope cassette is inserted into the ER and subsequently processed by host signalases (S) and furin-like proteases (F). *Blue chains* indicate N-glycosylation sites. Adapted from (Kuhn 2007a)

For the alphavirus type species Sindbis virus it has been shown that glycosylation does not necessary influence processing of PE2 or E1 but does influence virulence and viral replication (Knight et al. 2009).

Alphaviruses use low-pH triggered fusion to facilitate entry of target cells. This process is mediated by fusogenic active trimeric complexes at the surface of the virus particle. It would be detrimental for viral replication if the fusion protein is activated during the formation of the trimeric spike as it encounters the low-pH environment of the secretory pathway. The CHIKV fusion protein E1 is therefore expressed with the companion protein E2. The dimeric interaction between E1 and E2 protects the fusion protein from low-pH-induced premature activation (Uchime et al. 2013). The late secretory pathway involves compartments with a pH ranging from ~5.5 to 6.0, which is sufficient to trigger premature E1 inactivation and fusion (Uchime et al. 2013). However, the immature PE2-E1 dimer is more acid stable than the fully mature E2-E1 form.

E3 plays a critical role in the stabilization and protection of the immature trimer from acidic environments. After initial processing and dimerization, the small peripheral E3 protein is finally released from PE2 by furin-dependent cleavage in the *trans*-Golgi system (Strauss and Strauss 1994). Following cleavage, E3 remains noncovalently associated with the fully processed trimeric spike and is released upon spike surface exposure at neutral pH. Furin processing is not a precondition for CHIKV virion assembly, but incomplete processing results in impaired fusion activity of the immature trimeric spikes (Strauss and Strauss 1994). Crystal structures of both PE2-E1 and E2-E1 heterodimers show that E3 exclusively interacts with E2, suggesting a dimer-stabilizing mechanism to protect E1 from premature activation before it reaches the cell surface (Li et al. 2010; Voss et al. 2010). Mutational studies have shown that the pH-protective interaction between E3 and E2 is highly dependent on a single amino acid (Y47) within the E3–E2 interface (Uchime et al. 2013).

CHIKV has been shown to be very suitable for the generation of so-called virus-like particles (VLPs). These artificial viral particles are replication defective due to absence of genomic viral RNA, but share the morphological properties of wild-type virus (Fig. 3). This means that VLP formation most likely shares all characteristics and processing kinetics found during wild-type CHIKV replication.

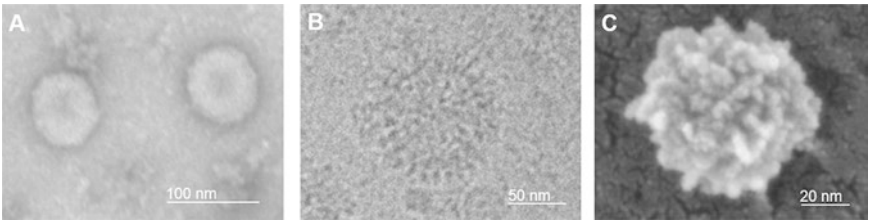

Fig. 3 CHIKV virus-like particles produced in insect cells. (**a**) CHIKV VLPs visualized by transmission electron microscopy after negative stain, (**b**) cryo-electron microscopy, and (**c**) scanning electron microscopy

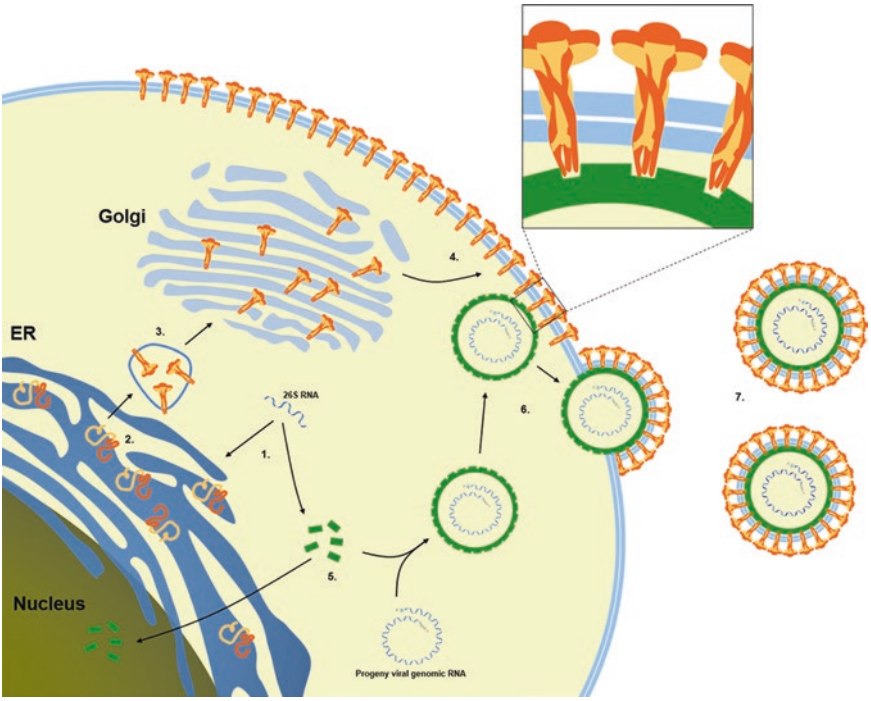

Fig. 4 CHIKV virus particle formation and maturation. The CHIKV structural proteins are translated from the 26S RNA (*1*) after which the envelope glycoproteins E1 and E2 are transported to the ER, complex into heterodimers (*2*) and are transported to the Golgi (*3*) where three heterodimers complex into trimeric spikes and furin processing takes place. (*4*) The mature trimeric spikes are transported to the plasma membrane and are exposed on the surface of the cell. The capsid protein interacts with the progeny viral genomic RNA and assembles into nucleocapsids in the cytoplasm (*5*). The nucleocapsids bud out from the plasma membrane, taking along the trimeric spikes anchored in the lipid bilayer (*6*), resulting in mature CHIKV particles (*7*)

For the production of CHIKV VLPs, the complete structural polyprotein is expressed within a mammalian or insect cell expression system to allow correct poly(glyco)protein processing and trimer formation (Fig. 4) (Akahata et al. 2010; Metz et al. 2013a; Noranate et al. 2014).

Interestingly, different CHIKV strains yield variable quantities of VLPs. It has been shown that particle assembly and release are related to factors such as palmitoylation of the E1 and E2, cholesterol requirements, and pH-levels. A so-called acid-sensitive region (ASR) has been identified in E2, which is known to initiate conformational changes in the E1–E2 complex. Mutational changes of especially amino acid 234 in E2, or changes in pH increased VLP production yields.

In many neutralizing antibody escape mutants, modifications of the E2 B-domain have been found, in addition to modifications in the E2-ASR. Specific amino acid substitutions have been identified that prevent antibodies from neutralizing the virus by binding to amino acids that regulate the conformational changes to prime to

fusion complex active (Coffey and Vignuzzi 2011). All these characteristics can be used to optimize VLP production yields, especially because CHIKV-VLPs are considered to be among the most promising vaccine candidates in development (Akahata et al. 2010; Metz et al. 2013a, b).

Accessory Proteins 6K and Transframe Protein TF

For decades it was believed that there were only five structural proteins encoded by CHIKV (C, E3, E2, 6K, and E1) and other alphavirus species. 6K is a small, hydrophobic acylated protein that is involved in membrane permeabilization and envelope development but rarely is incorporated into mature virions (Antoine et al. 2007; Firth et al. 2008; Strauss and Strauss 1994; Welch and Sefton 1979). The C-terminal domain of 6K acts as an ER-translocation signal for E1. However, 6K deletion mutants have shown that E1 still localizes to the ER by the N-terminal signal sequence of PE2 (Liljestrom and Garoff 1991). Thus, envelope glycoprotein translocation can act independently of 6K (Metz et al. 2011; Strauss and Strauss 1994).

In the ER, 6K is processed by host signalases and released from PE2 and E1 after which 6K becomes associated with the PE2–E1 complex and is transported with the complex to the cell surface. Yet, during virus budding, 6K is mostly excluded from integration into new virons (Lusa et al. 1991). Even though 6K deletion mutants are still viable, mutations in 6K are associated with decreased virion production with impaired fusion activity and core deformations (Antoine et al. 2007; Firth et al. 2008; Gaedigk-Nitschko et al. 1990; Gaedigk-Nitschko and Schlesinger 1990; Loewy et al. 1995). Several studies have shown the importance of 6K in virus budding. It is postulated that 6K allows lipids from the membrane to flip from one side of the bilayer to the other (Gaedigk-Nitschko et al. 1990). With the use of chimeric alphaviruses it was found that 6K interacts in a sequence-specific manner with PE2 or E1. This interaction is required for efficient virus budding because 6K proteins are not interchangeable between alphavirus species (Yao et al. 1996).

Early observations report that the 6K migrated as a doublet, and differences found in protein size and biochemical properties were explained by different levels of acetylation. Recent analyses of this 6K doublet and its coding region resulted in the identification of a sixth structural protein. This so-called transframe protein (TF) originates from a ribosomal −1 frameshifting event at the C-terminus of the 6K coding region (Firth et al. 2008). This frameshifting occurs at an estimated efficiency of approximately 10–18 % and takes place at a conserved UUUUUUA motif within the 6K coding sequence. The resulting TF of approximately 8 kDa in size shares its N-terminus with 6K, but lacks the second transmembrane region found in 6K. The TF C-terminus is encoded by the −1 frame. Additional alphavirus 3′sequences involved in efficient frameshifting have a remarkable diversity (Chung et al. 2010).

6K has been shown to possess viroporin properties, a small protein that is able to increase membrane permeability that favors virion budding. Interestingly and other than 6K, TF has transmembrane domain flanking regions that are rich in basic resi-

dues, which is characteristic for several other viroporins. Thus, TF may be the actual viroporin and therefore important for virus budding (Firth et al. 2008). Further analysis revealed, though in rare occasions, that not 6K but TF is predominantly incorporated in viral particles, suggesting a role in the formation and budding of new virions (Firth et al. 2008).

Similar to 6K, TF is not absolutely required for genome replication or envelope protein translocation to the cell surface, but abolishing its production severely decreases virus particle release in both mammalian and insect cell systems (Snyder et al. 2013). Even though the precise mechanisms underlying their roles in membrane permeabilization, ion-gradient formation, and virus assembly and release are not yet fully understood, 6K and TF are both critical players in the late stages of CHIKV virion formation.

Role of CHIKV Structural Proteins During Viral Entry and Fusion

In general, alphaviruses are able to infect a wide range of species and cell types, because the viruses are most likely able to recognize and bind a range of different receptors on the host cell surface (Kielian et al. 2010). The process of particle attachment and absorption is a multistep event orchestrated by CHIKV-E1 and E2. Being an arbovirus, CHIKV infects both insect and vertebrate cells, meaning that the virus needs to deal with a wide range of divergent biochemical and genetic environments. Thus it is likely that CHIKV uses ubiquitous receptors and/or is able to bind multiple (protein) receptors (Kononchik et al. 2011).

Virus infection starts with scanning cell surfaces to encounter one or more suitable receptors. Subsequent binding of the virus is saturable and is primarily mediated by E2 (Kielian et al. 2010). Even though the exact receptors have not been identified yet, several proteinaceous or polysaccharide molecules have been suggested to be attachment factors for alphaviruses. The list includes the high affinity laminin receptor (Wang et al. 1992), heparin and heparan sulfate (Byrnes and Griffin 1998; Smit et al. 2002; Zhang et al. 2005), heat shock 70 protein (Ryan et al. 1992), the major histocompatibility complex (Helenius et al. 1978; Maassen and Terhorst 1981), and DC-SIGN and L-SIGN (Klimstra et al. 2003). The latter ones are C-type lectins that have high binding affinity for mannose-rich carbohydrate structures. Interestingly, mosquito cells produce these high mannose structures and mosquito-derived virus shows increased binding and infection on DC-SIGN/L-SIGN expressing cells. Thus, a high mannose glycan state of E2 might have a strong influence on its ability to bind receptor-associated molecules (Klimstra et al. 2003).

Upon receptor binding by E2, conformational changes within the trimeric spike and the mild acidic endosomal environment trigger the dissociation of the E1–E2 heterodimer and induce a homotrimerization event between the three E1 molecules. CHIKV-E1 is a type II fusion protein and its ectodomain is composed of three domains: the central domain (DI), the fusion loop containing domain (DII), and

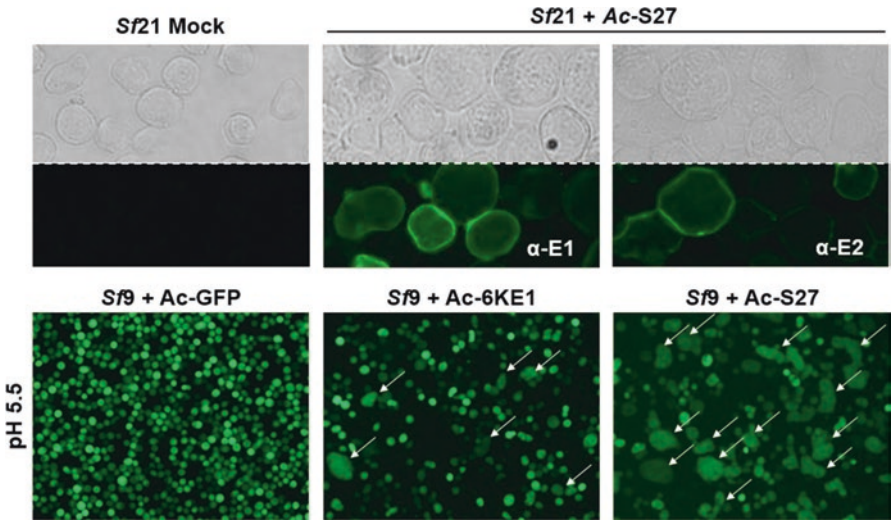

Fig. 5 CHIKV glycoprotein expression and fusogenicity. The complete structural protein cassette of CHIKV was expressed in insect cells using recombinant baculoviruses. (**a**) Healthy and infected Sf-21 insect cells were immunostained with E1 and E2 specific antibodies. The clear ring-like structures indicate surface expression of the glycoproteins. (**b**) Individual E1 and the complete structural cassette of CHIKV were expressed in GFP-expressing insect cells. At a pH of 5.5, the fusion protein E1 is activated, resulting in the fusion of proximate cell membranes known as syncytia formation

domain III (DIII), which connects to the transmembrane domain (TM; Sanchez-San Martin et al. 2013). The E1 fusion loop is located at the tip of DII and is normally shielded in the E1–E2 interface by interaction with histidine residues in a so-called fusion loop binding groove within E2 (Voss et al. 2010). During fusion, E1 is inserted into the host's plasma membrane through the fusion loop. A core trimer is formed by interactions between DI and DII, forming a hairpin-like structure by which DIII is able to pack against the core trimer. This conformational reassortment brings the viral and target membrane in close proximity enabling membrane fusion (Gibbons et al. 2004). Even though the subtle interactions between E1 and E2 are critical in the formation of the surface trimers, E1 on itself remains fusogenic (Metz et al. 2011). When E1 is expressed in a 6KE1 context in insect cells, syncytia are formed between E1-expressing cells that display E1 on the cells' surface (Fig. 5).

In the past decade, more evidence has accumulated that supposes an alternative entry mechanism for alphaviruses independent of endocytosis, exposure to low pH, and membrane fusion. E1-mediated membrane fusion is a so-called nonleaky process where viruses are taken up into the cell without losing plasma membrane continuity. The alternative entry mechanism is supported by the fact that infection by alphaviruses appears to be a leaky process that allows the passage of ions and small molecules across the compromised membrane (Kononchik et al. 2011; Koschinski et al. 2005; Madan et al. 2005; Wengler et al. 2003, 2004). Such ion-permeable pores are believed to be formed by E1 insertions into the viral or E1-expressing cell

membrane, in the absence of a target membrane (Koschinski et al. 2005). The importance of these ion-permeable pores in infection is still being discussed, yet again it shows the critical role of E1 in virus entry.

In addition, research is now focusing on the potential role of 6K or TF in the pore entry pathway. Even though the two small proteins are rarely incorporated in the virus particle, they are able to generate plasma membrane pores late in infection and 6K/TF deletion mutants generate viable, but fusogenically compromised virus particles (Sanz et al. 2003).

References

Akahata W, Yang ZY, Andersen H, Sun S, Holdaway HA, Kong WP, Lewis MG, Higgs S, Rossmann MG, Rao S, Nabel GJ (2010) A virus-like particle vaccine for epidemic chikungunya virus protects nonhuman primates against infection. Nat Med 16(3):334–338. doi:10.1038/nm.2105, nm.2105 [pii]

Aliperti G, Schlesinger MJ (1978) Evidence for an autoprotease activity of sindbis virus capsid protein. Virology 90(2):366–369

Antoine AF, Montpellier C, Cailliau K, Browaeys-Poly E, Vilain JP, Dubuisson J (2007) The alphavirus 6K protein activates endogenous ionic conductances when expressed in Xenopus oocytes. J Membr Biol 215(1):37–48. doi:10.1007/s00232-007-9003-6

Blom N, Sicheritz-Pontén T, Gupta R, Gammeltoft S, Brunak S (2004) Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. Proteomics 4(6):1633–1649

Burke D, Keegstra K (1979) Carbohydrate structure of sindbis virus glycoprotein E2 from virus grown in hamster and chicken cells. J Virol 29(2):546–554

Byrnes AP, Griffin DE (1998) Binding of Sindbis virus to cell surface heparan sulfate. J Virol 72(9):7349–7356

Choi HK, Tong L, Minor W, Dumas P, Boege U, Rossmann MG, Wengler G (1991) Structure of Sindbis virus core protein reveals a chymotrypsin-like serine proteinase and the organization of the virion. Nature 354(6348):37–43. doi:10.1038/354037a0

Chung BY, Firth AE, Atkins JF (2010) Frameshifting in alphaviruses: a diversity of 3′ stimulatory structures. J Mol Biol 397(2):448–456. doi:10.1016/j.jmb.2010.01.044

Coffey LL, Vignuzzi M (2011) Host alternation of chikungunya virus increases fitness while restricting population diversity and adaptability to novel selective pressures. J Virol 85(2):1025–1035. doi:10.1128/JVI.01918-10

Firth AE, Chung BY, Fleeton MN, Atkins JF (2008) Discovery of frameshifting in Alphavirus 6K resolves a 20-year enigma. Virol J 5:108. doi:10.1186/1743-422X-5-108

Gaedigk-Nitschko K, Schlesinger MJ (1990) The Sindbis virus 6K protein can be detected in virions and is acylated with fatty acids. Virology 175(1):274–281

Gaedigk-Nitschko K, Ding MX, Levy MA, Schlesinger MJ (1990) Site-directed mutations in the Sindbis virus 6K protein reveal sites for fatty acylation and the underacylated protein affects virus release and virion structure. Virology 175(1):282–291

Garmashova N, Gorchakov R, Volkova E, Paessler S, Frolova E, Frolov I (2007) The Old World and New World alphaviruses use different virus-specific proteins for induction of transcriptional shutoff. J Virol 81(5):2472–2484. doi:10.1128/JVI.02073-06

Gibbons DL, Vaney MC, Roussel A, Vigouroux A, Reilly B, Lepault J, Kielian M, Rey FA (2004) Conformational change and protein-protein interactions of the fusion protein of Semliki Forest virus. Nature 427(6972):320–325. doi:10.1038/nature02239

Hahn CS, Strauss JH (1990) Site-directed mutagenesis of the proposed catalytic amino acids of the Sindbis virus capsid protein autoprotease. J Virol 64(6):3069–3073

Helenius A, Morein B, Fries E, Simons K, Robinson P, Schirrmacher V, Terhorst C, Strominger JL (1978) Human (HLA-A and HLA-B) and murine (H-2K and H-2D) histocompatibility antigens are cell surface receptors for Semliki Forest virus. Proc Natl Acad Sci U S A 75(8):3846–3850

Hong EM, Perera R, Kuhn RJ (2006) Alphavirus capsid protein helix I controls a checkpoint in nucleocapsid core assembly. J Virol 80(18):8848–8855. doi:10.1128/JVI.00619-06

Khan AH, Morita K, Parquet Md Mdel C, Hasebe F, Mathenge EG, Igarashi A (2002) Complete nucleotide sequence of chikungunya virus and evidence for an internal polyadenylation site. J Gen Virol 83(Pt 12):3075–3084

Kielian M, Chanel-Vos C, Liao M (2010) Alphavirus entry and membrane fusion. Viruses 2(4):796–825. doi:10.3390/v2040796

Klimstra WB, Nangle EM, Smith MS, Yurochko AD, Ryman KD (2003) DC-SIGN and L-SIGN can act as attachment receptors for alphaviruses and distinguish between mosquito cell- and mammalian cell-derived viruses. J Virol 77(22):12022–12032

Knight RL, Schultz KL, Kent RJ, Venkatesan M, Griffin DE (2009) Role of N-linked glycosylation for sindbis virus infection and replication in vertebrate and invertebrate systems. J Virol 83(11):5640–5647

Kononchik JP Jr, Hernandez R, Brown DT (2011) An alternative pathway for alphavirus entry. Virol J 8:304. doi:10.1186/1743-422X-8-304

Koschinski A, Wengler G, Wengler G, Repp H (2005) Rare earth ions block the ion pores generated by the class II fusion proteins of alphaviruses and allow analysis of the biological functions of these pores. J Gen Virol 86(Pt 12):3311–3320. doi:10.1099/vir.0.81096-0

Kuhn RJ (2007a) Togaviridae: the viruses and their replication. Lippincott, Williams and Wilkins, Philadelphia, PA, pp 1001–1022

Kuhn RJ (2007b) Togaviridae: the viruses and their replication. In: Howley PM, Knipe DM (eds) Fields virology, 5th edn. Lippincott-Raven Publishers, Philadelphia, pp 1001–1022

Li L, Jose J, Xiang Y, Kuhn RJ, Rossmann MG (2010) Structural changes of envelope proteins during alphavirus fusion. Nature 468(7324):705–708. doi:10.1038/nature09546, nature09546 [pii]

Liljestrom P, Garoff H (1991) Internally located cleavable signal sequences direct the formation of Semliki Forest virus membrane proteins from a polyprotein precursor. J Virol 65(1):147–154

Lobigs M, Zhao HX, Garoff H (1990) Function of Semliki Forest virus E3 peptide in virus assembly: replacement of E3 with an artificial signal peptide abolishes spike heterodimerization and surface expression of E1. J Virol 64(9):4346–4355

Loewy A, Smyth J, von Bonsdorff CH, Liljestrom P, Schlesinger MJ (1995) The 6-kilodalton membrane protein of Semliki Forest virus is involved in the budding process. J Virol 69(1):469–475

Lusa S, Garoff H, Liljestrom P (1991) Fate of the 6K membrane protein of Semliki Forest virus during virus assembly. Virology 185(2):843–846

Maassen JA, Terhorst C (1981) Identification of a cell-surface protein involved in the binding site of Sindbis virus on human lymphoblastic cell lines using a heterobifunctional cross-linker. Eur J Biochem 115(1):153–158

Madan V, Sanz MA, Carrasco L (2005) Requirement of the vesicular system for membrane permeabilization by Sindbis virus. Virology 332(1):307–315. doi:10.1016/j.virol.2004.11.001

Melancon P, Garoff H (1987) Processing of the Semliki Forest virus structural polyprotein: role of the capsid protease. J Virol 61(5):1301–1309

Metz SW, Pijlman GP (2011) Arbovirus vaccines; opportunities for the baculovirus-insect cell expression system. J Invertebr Pathol 107(Suppl):S16–S30. doi:10.1016/j.jip.2011.05.002

Metz SW, Geertsema C, Martina BE, Andrade P, Heldens JG, van Oers MM, Goldbach RW, Vlak JM, Pijlman GP (2011) Functional processing and secretion of chikungunya virus E1 and E2 glycoproteins in insect cells. Virol J 8:353–365. doi:10.1186/1743-422X-8-353

Metz S, Gardner IA, Geertsema C, Thuy L, Goh L, Suhrbier A, Vlak J, Pijlman G (2013a) Effective chikungunya virus-like particle vaccine produced in insect cells. PLoS Negl Trop Dis 7(3):e2124

Metz SW, Martina BE, van den Doel P, Geertsema C, Osterhaus AD, Vlak JM, Pijlman GP (2013b) Chikungunya virus-like particles are more immunogenic in a lethal AG129 mouse model compared to glycoprotein E1 or E2 subunits. Vaccine 31(51):6092–6096. doi:10.1016/j.vaccine.2013.09.045

Noranate N, Takeda N, Chetanachan P, Sittisaman P, A-Nuegoonpipat A, Anantapreecha S (2014) Characterization of chikungunya virus-like particles. PLoS One 9(9):e108169. doi:10.1371/journal.pone.0108169

Owen KE, Kuhn RJ (1996) Identification of a region in the Sindbis virus nucleocapsid protein that is involved in specificity of RNA encapsidation. J Virol 70(5):2757–2763

Perera R, Owen KE, Tellinghuisen TL, Gorbalenya AE, Kuhn RJ (2001) Alphavirus nucleocapsid protein contains a putative coiled coil alpha-helix important for core assembly. J Virol 75(1):1–10. doi:10.1128/JVI.75.1.1-10.2001

Rice CM, Strauss JH (1981) Nucleotide sequence of the 26S mRNA of Sindbis virus and deduced sequence of the encoded virus structural proteins. Proc Natl Acad Sci U S A 78(4):2062–2066

Ryan C, Stevens TH, Schlesinger MJ (1992) Inhibitory effects of HSP70 chaperones on nascent polypeptides. Protein Sci 1(8):980–985. doi:10.1002/pro.5560010803

Sanchez-San Martin C, Nanda S, Zheng Y, Fields W, Kielian M (2013) Cross-inhibition of chikungunya virus fusion and infection by alphavirus E1 domain III proteins. J Virol 87(13):7680–7687. doi:10.1128/JVI.00814-13

Sanz MA, Madan V, Carrasco L, Nieva JL (2003) Interfacial domains in Sindbis virus 6K protein. Detection and functional characterization. J Biol Chem 278(3):2051–2057. doi:10.1074/jbc.M206611200

Simizu B, Yamamoto K, Hashimoto K, Ogata T (1984) Structural proteins of chikungunya virus. J Virol 51(1):254–258

Smit JM, Waarts BL, Kimata K, Klimstra WB, Bittman R, Wilschut J (2002) Adaptation of alphaviruses to heparan sulfate: interaction of Sindbis and Semliki forest viruses with liposomes containing lipid-conjugated heparin. J Virol 76(20):10128–10137

Snyder JE, Kulcsar KA, Schultz KL, Riley CP, Neary JT, Marr S, Jose J, Griffin DE, Kuhn RJ (2013) Functional characterization of the alphavirus TF protein. J Virol 87(15):8511–8523. doi:10.1128/JVI.00449-13

Strauss JH, Strauss EG (1994) The alphaviruses: gene expression, replication, and evolution. Microbiol Rev 58(3):491–562

Thomas S, Rai J, John L, Schaefer S, Putzer BM, Herchenroder O (2013) Chikungunya virus capsid protein contains nuclear import and export signals. Virol J 10:269. doi:10.1186/1743-422X-10-269

Uchime O, Fields W, Kielian M (2013) The role of E3 in pH protection during alphavirus assembly and exit. J Virol 87(18):10255–10262. doi:10.1128/JVI.01507-13

van den Doel P, Volz A, Roose JM, Sewbalaksing VD, Pijlman GP, van Middelkoop I, Duiverman V, van de Wetering E, Sutter G, Osterhaus AD, Martina BE (2014) Recombinant modified vaccinia virus Ankara expressing glycoprotein E2 of chikungunya virus protects AG129 mice against lethal challenge. PLoS Negl Trop Dis 8(9):e3101. doi:10.1371/journal.pntd.0003101

Voss JE, Vaney MC, Duquerroy S, Vonrhein C, Girard-Blanc C, Crublet E, Thompson A, Bricogne G, Rey FA (2010) Glycoprotein organization of chikungunya virus particles revealed by X-ray crystallography. Nature 468(7324):709–712. doi:10.1038/nature09555, nature09555 [pii]

Wang KS, Kuhn RJ, Strauss EG, Ou S, Strauss JH (1992) High-affinity laminin receptor is a receptor for Sindbis virus in mammalian cells. J Virol 66(8):4992–5001

Weiss B, Nitschko H, Ghattas I, Wright R, Schlesinger S (1989) Evidence for specificity in the encapsidation of Sindbis virus RNAs. J Virol 63(12):5310–5318

Welch WJ, Sefton BM (1979) Two small virus-specific polypeptides are produced during infection with Sindbis virus. J Virol 29(3):1186–1195

Wengler G, Koschinski A, Wengler G, Dreyer F (2003) Entry of alphaviruses at the plasma membrane converts the viral surface proteins into an ion-permeable pore that can be detected by electrophysiological analyses of whole-cell membrane currents. J Gen Virol 84(Pt 1):173–181

Wengler G, Koschinski A, Wengler G, Repp H (2004) During entry of alphaviruses, the E1 glycoprotein molecules probably form two separate populations that generate either a fusion pore or ion-permeable pores. J Gen Virol 85(Pt 6):1695–1701

Yao JS, Strauss EG, Strauss JH (1996) Interactions between PE2, E1, and 6K required for assembly of alphaviruses studied with chimeric viruses. J Virol 70(11):7910–7920

Zhang W, Heil M, Kuhn RJ, Baker TS (2005) Heparin binding sites on Ross River virus revealed by electron cryo-microscopy. Virology 332(2):511–518. doi:10.1016/j.virol.2004.11.043